Effects of aging, high-fat diet, and testosterone treatment on neural and metabolic outcomes in male brown Norway rats

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ABSTRACT

Risk for Alzheimer's disease (AD) is affected by multiple factors, including aging, obesity, and low testosterone. We previously showed that obesity and low testosterone independently and interactively exacerbate AD-related outcomes in young adult rodents. The goals of the present study are two-fold: to examine whether the effects of an obesogenic diet differ with increasing age and to determine if testosterone treatment in middle-aged and aged animals mitigates negative effects of the diet. Male brown Norway rats were maintained on control or high-fat diets for 12 weeks beginning in young adulthood, middle age, or advanced age. Separate cohorts of middle-aged and aged animals were treated with testosterone during dietary manipulations. Endpoints included metabolic indices, inflammation, cognitive performance, and neural health outcomes. Aging was associated with poorer outcomes that were generally exacerbated by high-fat diet, especially at middle age. Testosterone treatment was largely without benefit, exerting only subtle effects on a select number of measures. Understanding how the deleterious effects of obesity are affected by advancing age and the ability of protective strategies such as testosterone to reduce these effects may provide significant insight into both the development and prevention of age-related cognitive decline and AD.

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1. Introduction

Obesity is a growing public health concern that increases risks for death and several diseases, including type 2 diabetes, cardio-vascular disease, and various cancers (Zheng et al., 2017). Obesity is also associated with numerous adverse neural outcomes (Asih et al., 2017; Jayaraman and Pike, 2014; Lee and Mattson, 2014). For example, obesity is linked with decreases in hippocampal volume and white matter integrity (Ho et al., 2010; Jagust et al., 2005; Stanek et al., 2011) as well as accelerated cognitive decline (Cournot et al., 2006; Elias et al., 2005). Importantly, obesity also increases up to three-fold the risk of dementias (Whitmer et al., 2008), including Alzheimer's disease (AD) (Profenno et al., 2010) and vascular dementia (Hayden et al., 2006).

Although advanced age is the single greatest risk factor for cognitive decline and dementia (Niccoli and Partridge, 2012),

whether and how aging affects neural outcomes of obesity is not well understood. Weight gain increases during adulthood (Sheehan et al., 2003) leading to rates of obesity that peak at late middle age (Mizuno et al., 2004) and are associated with a wide range of serious health outcomes (Zheng et al., 2017). Several studies indicate that obesity during middle age is especially harmful, increasing rates of cognitive decline (Bischof and Park, 2015; Cournot et al., 2006; Dahl et al., 2013) and risk of dementia (Emmerzaal et al., 2015; Fitzpatrick et al., 2009) later in life. In animal studies, diet-induced obesity (DIO) is associated with reduced hippocampal neurogenesis (Lindqvist et al., 2006; Park et al., 2010), cognitive impairment (Kanoski et al., 2010; Knight et al., 2014; Stranahan et al., 2008), and increased AD-related pathology (Barron et al., 2013; Ho et al., 2004; Julien et al., 2010; Kohjima et al., 2010). The role of aging in the neural effects of DIO has only been addressed by a limited number of studies (Boitard et al., 2012; Erdos et al., 2011; Larkin et al., 2001; Spencer et al., 2017).

One normal physiological change that occurs in middle-aged men and is linked with the development of both obesity (Zitzmann, 2009) and dementia (Pike et al., 2009) is a decrease in testosterone levels. Importantly, there appears to be a bidirectional

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relationship between testosterone loss and obesity, in that loss of testosterone can increase adiposity and risk for obesity, and obesity, in turn, can decrease testosterone levels (De Maddalena et al., 2012; Fui et al., 2014). Because testosterone exerts a number of neural benefits, including increased hippocampal neurogenesis, neuroprotection, improved performance in select cognitive tasks, and reduction of AD-related neuropathology (Galea, 2008; Rosario and Pike, 2008), age-related testosterone depletion also may be expected to affect several neural consequences related to obesity. Consistent with this possibility, our prior work in male rodents identified interactions between testosterone and obesity in terms of both metabolic and neural outcomes (Jayaraman et al., 2014). Such findings suggest that testosterone therapy may provide a valuable approach to reduce negative effects of obesity during aging. Indeed, testosterone therapy has been reported to improve body composition (Isidori et al., 2005) by reducing body weight, body mass index (BMI), symptoms of metabolic syndrome (Traish et al., 2014; Yassin et al., 2014) and visceral adiposity, while increasing skeletal muscle (Allan et al., 2008). However, a recent meta-analysis of randomized clinical trials reported only limited net benefits of testosterone (Ponce et al., 2018). Furthermore, new findings from the Testosterone Trials show no significant cognitive improvement (Resnick et al., 2017) and only modest metabolic benefits (Snyder et al., 2018) in aging, hypogonadal men treated with testosterone. The mixed evidence of testosterone treatment underscores the need for greater understanding of the contexts and outcomes for which testosterone therapy may have significant efficacy.

The available literature suggests that advancing age and obesity may act not only individually, but also cooperatively to drive a number of adverse outcomes. The extent to which protective actions of testosterone against obesity may offer a therapeutic approach to minimize the deleterious consequences of obesity during aging is unclear. To investigate these interactions, we conducted two parallel studies in male brown Norway rats, which have been demonstrated to model age-related testosterone loss in men (Gruenewald et al., 2008; Wang et al., 1993, 2002). Our goal was to examine the effects of high-fat diet (HFD) across the lifespan and the role of testosterone in this relationship. Thus, we compared, in two separate experiments, the effects of both age and testosterone supplementation on metabolic, cognitive, inflammatory, and neural health outcomes associated with HFD. First, we examined the effects of aging by comparing HFD outcomes in male rats at young adulthood (3 months), middle age (13 months), and advanced age (23 months). In the second experiment, we evaluated whether testosterone supplementation alters the effects of HFD in middle-aged and aged animals that are vulnerable to normal, age-related testosterone depletion.

2. Methods

2.1. Animal procedures

2.1.1. Experiment 1
2.1.1.1. Aging and diet.
Male brown Norway rats aged 3 (young), 13 (middle-aged), and 23 (aged) months were provided by the Aged Rodent Colony at the National Institute on Aging and subsequently maintained at vivarium facilities at University of Southern California. All animals were sexually naïve. Throughout the experimental period, animals were housed under a 12-hour light/dark cycle with lights on at 6 AM and had *ad libitum* access to food and water. After a 1-week gap to allow animals to acclimate to the room in which they were housed, they were randomized to one of two dietary treatment groups: control diet (10% fat; #D12450J, Research Diets, Inc., New Brunswick, NJ, USA; CTL) or HFD (60% fat; #D12492, Research Diets, Inc.; HFD). The animals were kept on experimental

diets for 12 weeks during which body weight (expressed as both actual body weight and % change in body weight) and food consumption (expressed as mean kcal consumed per day) were recorded weekly.

At the end of the treatment period, rats were euthanized with inhalant carbon dioxide, and the brains were rapidly removed. One hemibrain was immersion-fixed for 48 hours in 4% paraformaldehyde/0.067 M Sorensen's phosphate buffer and then stored at 4 °C in 0.1 M phosphate buffered saline/0.03% NaN₃ until processed for immunohistochemistry. The remaining hemibrain was snap frozen and then stored at −80 °C before processing for RNA extraction. Blood was collected in EDTA-coated tubes via cardiac puncture and centrifuged to separate plasma, which was aliquoted and stored at −80 °C. The liver was dissected and snap frozen for RNA extraction. Gonadal and retroperitoneal fat were dissected and weighed as a measure of adiposity.

2.1.2. Experiment 2
2.1.2.1. Testosterone and diet.
To examine the effects of testosterone supplementation on diet-associated outcomes during aging, we enrolled additional groups of 13-month-old (middle-aged) and 23-month-old (aged) brown Norway rats that were treated with testosterone and randomly assigned to either CTL or HFD. Rats were housed under the same conditions as described previously. Testosterone treatment was initiated simultaneously with dietary treatments. Animals were anesthetized with inhalant isoflurane and subcutaneously implanted between the shoulder blades with a silastic capsule (1.57 mm ID × 3.18 mm OD; Dow Corning, Midland, MI, USA) that was a total length of 2 cm with the inner 1 cm packed with testosterone (Steraloids, Newport, RI, USA). Capsules were removed and replaced with fresh testosterone-containing capsules at the 6-week time point. Dosing was based on previous studies showing that this treatment results in physiological testosterone levels (Edinger and Frye, 2004; Frye et al., 2010; Pinilla et al., 1999). The highly androgen-responsive tissues levator ani muscle, seminal vesicles, and prostrate were collected and weighed as established bioassays to confirm efficacy of testosterone treatment (Owens et al., 2006). All animal procedures were carried out under a protocol approved by the University of Southern California Institutional Animal Care and Use Committee and in accordance with the National Institute of Health standards.

2.2. Glucose homeostasis

A glucose tolerance test was performed at week 11. After a 16-hour overnight fast, baseline glucose levels were recorded, and rats were administered a glucose bolus (2 g/kg body weight) via intraperitoneal injection. Blood glucose levels were recorded 15, 30, 60, and 120 minutes after administration of the glucose bolus. Blood was collected from the lateral tail vein and immediately assessed for glucose levels using the Precision Xtra Blood Glucose and Ketone Monitoring System (Abbott Diabetes Care, Alameda, CA, USA). The extent to which animals returned to their baseline glucose reading after 120 minutes and the total area under the curve (AUC) across 120 minutes were calculated.

2.3. Leptin and testosterone ELISAs

Plasma leptin levels were determined by ELISA using a commercially available kit (Rat Leptin ELISA, #EZRL-83K, Millipore, Burlington, MA, USA). All samples were run in duplicate according to manufacturer's instructions. Plasma samples were also assayed for testosterone using an adaptation of the ImmuChem Double Antibody testosterone RIA kit from MP Biomedicals (#07-189102, Costa Mesa, CA, USA) with [125I] testosterone as the tracer and all

reagent volumes halved. The testosterone antibody (solid phase) cross-reacts slightly with 5α-DHT (3.4%), 5 α-andros- tane-3β, 17β-diol (2.2%), and 11-oxotestosterone (2%) but does not cross-react with progesterone, estradiol, or the glucocorticoids (all <0.01%). The minimum detectable testosterone concentration was 0.1 ng/mL and the intra-assay coefficient of variation was 6.0%.

2.4. Immunohistochemistry and quantification

Fixed hemibrains were completely sectioned across the horizontal plane at 40 μm using a vibratome (Leica Biosystems, Buffalo Grove, IL, USA). Immunohistochemistry was performed using a standard avidin/biotin peroxidase approach with ABC Vector Elite kits (Vector Laboratories, Burlingame, CA, USA). Every 13th section was processed for glial fibrillary acidic protein (GFAP), ionized calcium-binding adaptor molecule 1 (IBA-1), and doublecortin (DCX). Briefly, for GFAP staining, sections were rinsed in Tris buffered saline (TBS) before being treated with an endogenous peroxidase blocking solution for 10 minutes. After three 10-minute washes in 0.1% Triton-X/TBS, sections were incubated for 30 minutes in a blocking solution consisting of 2% bovine serum albumin in TBS. Blocked sections were incubated overnight at 4 °C in primary antibody directed against GFAP (#Z0334, 1:10,000 dilution, Dako, Carpinteria, CA, USA) that was diluted in the blocking solution. Sections were then rinsed and incubated in the biotinylated secondary antibody diluted in the blocking solution. Immunoreactivity was visualized using 3,3′-diaminobenzidine (Vector Laboratories).

Immediately adjacent tissue sections were similarly immunostained for IBA-1 with the following slight modifications. First, a 0.2% Triton-X/TBS solution was used for rinses on day 1, and both the blocking solution and primary antibody solution contained 0.2% Triton-X. Second, sections were blocked for 60 minutes before being incubated overnight in IBA-1 (#019-19741, 1:500 dilution, Wako Chemicals, Richmond, VA, USA).

In addition, sections were stained for DCX as previously described (Brummelte and Galea, 2010). Briefly, sections were treated with 0.6% hydrogen peroxidase for 30 minutes and then incubated for 24 hours in primary antibody at 4 °C (#sc-8066, 1:1000 dilution, goat anti-doublecortin, Santa Cruz Biotechnology, Santa Cruz, CA, USA), that was diluted in phosphate buffered saline with 0.4% Triton-X and 3% normal rabbit serum. Sections were then incubated with secondary antibody (#BA-5000, 1:500 dilution, biotinylated rabbit anti-goat, Vector Laboratories) for 24 hours at 4 °C and with Avidin-Biotin complex (1:1000 avidin and 1:1000 biotin, Vector Laboratories) for 4 hours at room temperature, before being visualized using 3,3′-diaminobenzidine with 1.6% nickel (Vector Laboratories). Tissue was rinsed 3 times in phosphate buffered saline for 10 minutes each between each step. The tissue was mounted onto microscope slides, dehydrated, cleared with xylene, and cover-slipped with Permount (Fisher Scientific).

Microglia density and activation were quantified using live imaging (Olympus, BX50, CAST Grid software, Olympus, Tokyo, Japan) and a 40x objective. IBA-1 immunoreactive cells were scored as exhibiting either resting or reactive phenotypes based on morphological criteria, as described in previous studies (Ayoub and Salm, 2003; Moser and Pike, 2017). In brief, IBA-1—labeled cells were scored as resting (type 1) microglia if their cell bodies were spherical with numerous thin and highly ramified processes. Cells were scored as having an activated phenotype if they had either (1) enlarged, rod-shaped cell bodies with fewer and thicker processes (type 2) or (2) very few, short processes or no processes with or without filopodia (type 3). The hippocampus was quantified starting in CA1 where the pyramidal cell layer begins. Five alternating fields were scored in each of 3 brain sections for a total of 15 fields and an average of ∼170 cells per brain.

Astrocytes were visualized by GFAP immunostaining and scored based on the morphological phenotype. Digital images were captured using a 40X objective with an Olympus BX50 microscope equipped with a DP74 camera and cellSens software. Images of four alternating fields were taken starting in CA1 of the hippocampus where the pyramidal cell layer begins and repeated across 3 brain sections for a total of 12 images per brain. This approach yielded an average of ∼450 cells scored per brain. GFAP-immunoreactive cells were scored using NIH ImageJ 1.50i (National Institutes of Health, Rockville, MD, USA) with the cell counter plugin to mark cells as having either a resting or a reactive morphological phenotype, as previously described (Moser and Pike, 2017; Wilhelmsson et al., 2006). In brief, cells were scored as having a resting phenotype if they displayed normal-sized somas with typical, generally short projections. Conversely, GFAP-immunoreactive cells were scored as having a reactive phenotype when they exhibited enlarged cell bodies and large, thick processes.

DCX staining was quantified using a Nikon E600 light microscope (Nikon, Tokyo, Japan) with a 40X objective lens. Cells were counted throughout the granule cell layer and area measurements were made of the granule cell layer using NIH ImageJ. A total of 10 sections per brain were quantified. To obtain cell density, the estimated total number of DCX-positive cells was divided by the total area counted per brain.

2.5. RNA isolation and quantitative PCR

RNA extractions and polymerase chain reaction (PCR) were performed as previously described (Jayaraman et al., 2014; Moser and Pike, 2017). For RNA extractions, the liver, hippocampus, and hypothalamus were homogenized using the TRIzol reagent (Invitrogen Corporation, Carlsbad, CA, USA) following the manufacturer's protocol. The RNA pellet was then treated with RNase-free DNase I (Epicentre, Madison, WI, USA) for 30 minutes at 37 °C to remove any remaining DNA contamination, and a phenol-chloroform extraction was performed to isolate RNA. Purified RNA (1 μg) was used to reverse transcribe cDNA using the iScript cDNA synthesis system (Bio-Rad, Hercules, CA, USA). Real-time quantitative PCR was then run on the resulting cDNA using SsoAdvanced Universal SYBR Green Supermix (Bio-Rad) and a Bio-Rad CFX Connect Thermocycler. All samples were run in duplicates. PCR products were quantified by normalizing with corresponding β-actin expression levels using the ΔΔ-CT method to acquire relative mRNA levels. Hippocampus and hypothalamus were probed for levels of interleukin-1 beta (IL-1β), cluster of differentiation 68 (CD68), and GFAP. The liver was probed for levels of CD68, sterol regulatory element binding protein-1, and stearoyl-CoA desaturase. Primer pair sequences are shown in Table 1.

Table 1
Primer sequences

Target gene	Sequence
Interleukin-1β (IL-1β)	Forward: 5′-GACTTCACCATGGAACCCGT-3′
	Reverse: 5′-GGAGACTGCCCATTCTCGAC-3′
Cluster of differentiation 68 (CD68)	Forward: 5′-AAGCAGCACAGTGGACATT-3′
	Reverse: 5′-TTCCGCAACAGAAGCTTTG-3′
Glial fibrillary acidic protein (GFAP)	Forward: 5′-TCAATGCCGGCTTCAAAGA-3′
	Reverse: 5′-AGCGCCTTGTTTTGCTGTTC-3′
Sterol regulatory element binding protein-1 (SREBP1)	Forward: 5′-GCTCACAAAAGCAAATCACT-3′
	Reverse: 5′-GCGTTTCTACCACTTCAGG-3′
Stearoyl-CoA desaturase (SCD-1)	Forward: 5′-GGGAAAGTGAAGCGAGCAA-3′
	Reverse: 5′-GTGGTCGTGTAGGAACTGGAGA-3′
β-actin	Forward: 5′-AGCCATGTACGTAGCCATCC-3′
	Reverse: 5′-CTCTCAGCTGTGGTGGTGAA-3′

Gene targets for the PCR analyses are listed with their corresponding oligonucleotide sequences for the forward and reverse primers.

2.6. Behavioral testing: barnes maze

Spatial memory was assessed during week 10 of the treatment period, using a modified Barnes maze protocol (McLay et al., 1999). The maze was composed of a circular platform with 20 holes located around the border with an escape box located beneath one hole. The maze was housed inside an area isolated with black curtains on which visual cues were placed on each of the four walls. Rats were habituated to the behavioral testing room and to the maze 24 hours before the first training day. Specifically, after habituating to the room in their home cage for 30 minutes, they were placed on the maze in a vertical cylinder that they could not see out of for 3 minutes. The cylinder was then moved to guide the animal into the escape box for 1 minute before they were returned to their home cages. Animals were trained to locate the escape box over the following 4 days, with 3 trials per day, and a 15-minute intertrial interval during which they were returned to their home cage. During training trials, a bright light was directed above the maze, and static was played from a speaker below the maze. On each training day, rats were again habituated to the testing room for 30 minutes, before being placed in the cylinder on the maze for 10 seconds. The rats were then given 3 minutes to move about the maze freely and locate the escape box. The animal was given 1 minute in the box once it located it, before being returned to its home cage. If animals did not locate the box within 3 minutes, they were gently guided into it and allowed to remain inside for 1 minute. The maze was cleaned with 70% ethanol between each animal. Forty-eight h after the last training trial, rats were tested on the probe trial, in which the escape box was removed and rats were given 3 minutes to freely explore the maze.

The training and probe trials were recorded using Noldus Ethovision XT software (Leesburg, VA, USA). For training trials, the latency to locate the escape box was recorded, whereas for the probe trial, the number of correct hole investigations (defined as the hole where the escape box was previously located, as well as 1 hole on either side), errors, and the distance traveled to reach the correct hole for the first time were recorded.

2.7. Statistical analyses

All data are reported as the mean ± the standard error of the mean. Raw data were analyzed using Prism 7 (GraphPad Software, Inc., La Jolla, CA, USA). For experiment 1, data were analyzed using a 2×3 analysis of variance (ANOVA) that contained the variables of diet (CTL, HFD) and age (young, middle-aged, aged). For experiment 2, data were analyzed using a 2×2 ANOVA that contained the variables of diet (CTL, HFD) and age (middle-aged, aged). Experiments 1 and 2 were analyzed separately; groups from the two experiments were not directly compared. For the analyses of body weight and glucose tolerance, which are measured across time, repeated measures ANOVAs were conducted. In the case of significant main effects, planned comparisons between groups of interest were made using the Bonferroni correction. Significance was set at a threshold of $p < 0.05$. In addition, correlations between metabolic, inflammatory, and behavioral outcomes were performed using the Spearman correlation coefficient.

3. Results

3.1. Body weight and adiposity

3.1.1. Body weight and adiposity: diet and aging

To examine the independent and interactive effects of aging and HFD, we first analyzed changes in body weight across the 12-week treatment period (Fig. 1A–C). There was a significant effect of age
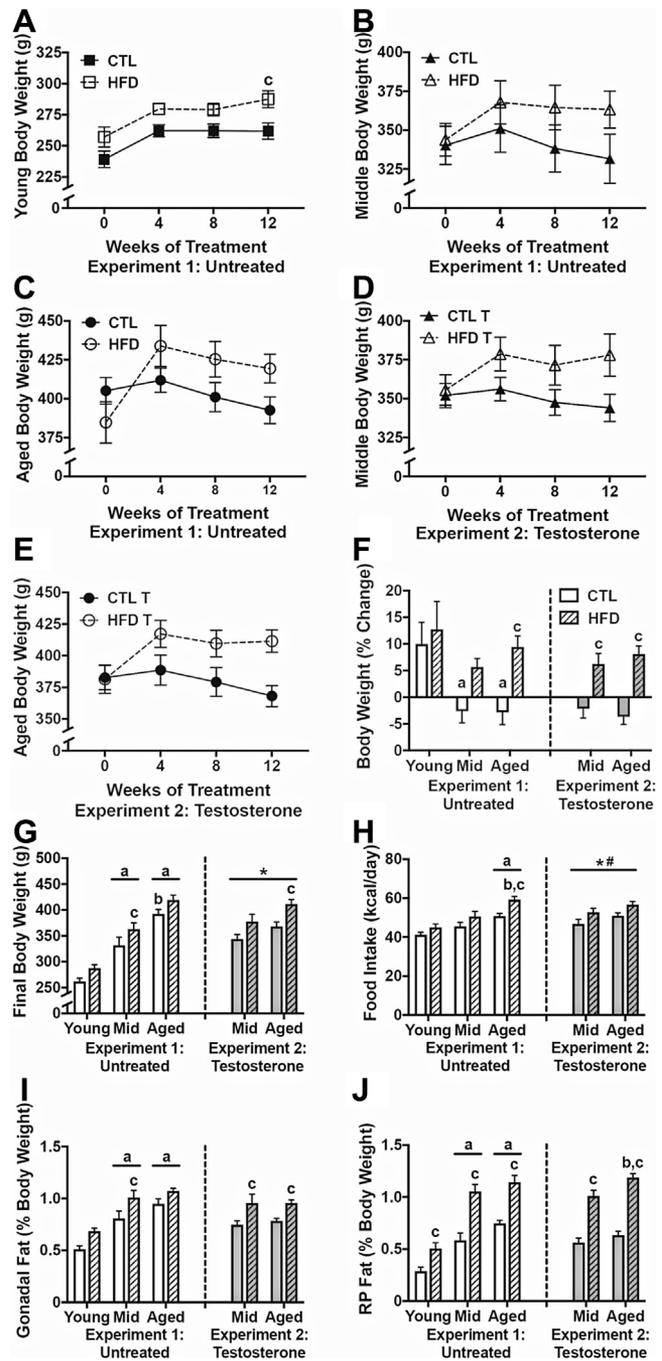

Fig. 1. Body weight and adiposity outcomes associated with high-fat diet in young, middle-aged, and aged brown Norway rats. (A-C) Body weights in (A) young, (B) middle-aged, and (C) aged male brown Norway rats maintained on control and high-fat diet taken at the baseline (week 0) and 4-week intervals across the 12-week diet treatment. (D-E) Body weights in testosterone-treated (D) middle-aged and (E) aged rats fed control or high-fat diet, across 12 weeks. (F) Percent change in body weight from the baseline to the end of the 12-week dietary treatment and (G) final body weight, in young, middle-aged, and aged male brown Norway rats after 12 weeks of high-fat diet feeding. (H) Food consumption as measured by the average daily kilocalories consumed across the 12-week dietary exposure. Adiposity is measured as weight of the (I) gonadal fat pads, and (J) retroperitoneal fat pads, relative to total body weight. Data are presented as mean (±standard error of the mean) values; n = 7–8/group. For figures A–E, young animals are shown as squares, middle-aged animals as triangles, and aged animals as circles; control diet are open symbols, high-fat diet are closed symbols. For figures F–H, animals not treated with testosterone are shown in white (experiment 1), and animals treated with testosterone in gray (experiment 2); control diets are solid white or gray bars, high-fat diets are striped bars. (a) $p < 0.05$ relative to young rats in same diet condition. (b) $p < 0.05$ relative to middle-aged animal in same diet condition. (c) $p < 0.05$ relative to control diet-fed of the same age. * $p < 0.05$ for main effect of age that does not reach statistical significance in post hoc tests. # $p < 0.05$ for main effect of diet that does not reach statistical significance in post hoc tests.

($F = 4.85$, $p = 0.003$) in which both middle-aged and aged animals weighed more than young animals, and aged animals weighed more than middle-aged animals at every time point ($p < 0.05$). There was also a significant effect of diet in the young animals ($F = 10.04$, $p = 0.007$; Fig. 1A). However, this effect reached statistical significance only at the 12-week time point ($p = 0.017$). There was no significant effect of diet on body weight at any time point in either middle-aged (Fig. 1B) or aged animals (Fig. 1C).

When examining the percent change in body weight from baseline to the end of the 12-week dietary treatment, we found significant effects of both age ($F = 5.65$, $p = 0.007$; Fig. 1F) and diet ($F = 9.34$, $p = 0.004$). Between-group comparisons revealed that, on control diet, young animals had a greater percent increase in body weight than both middle-aged ($p = 0.024$) and aged animals ($p = 0.021$), and that the effect of diet was significant only in aged animals ($p = 0.022$). Although comparisons between CTL and HFD failed to reach statistical significance when examined across time, we found a significant interaction effect between age and diet when comparing final body weights at 12 weeks ($F = 6.84$, $p = 0.003$; Fig. 1G), such that HFD only significantly increased body weight in middle-aged animals ($p < 0.001$). Moreover, there was a significant main effect of age ($F = 94.92$, $p < 0.001$), even in the absence of diet. Between-group comparisons revealed that middle-aged and aged animals weighed more than young rats on both CTL and HFD ($p < 0.01$), but aged animals weighed more than middle-aged animals only on a CTL diet ($p < 0.001$).

As differences in body weight can reflect, in part, differential food intake across age, we examined food intake, expressed as the average kilocalories consumed per day. We found a significant main effect of age on food intake ($F = 20.07$, $p < 0.0001$; Fig. 1H), with aged animals consuming more food than young animals on both control ($p = 0.003$) and HFD ($p < 0.0001$) diets, and more than middle-aged animals on HFD ($p = 0.006$). Moreover, there was a significant main effect of diet ($F = 14.06$, $p = 0.0005$), which was significant only in aged animals ($p = 0.008$).

In addition to increasing body weight, HFD is also associated with increased adiposity. Thus, we analyzed weights of the gonadal and retroperitoneal (RP) fat pads. Aging was associated with increases in weights of the gonadal ($F = 36.56$, $p < 0.001$; Fig. 1I) and RP ($F = 51.98$, $p < 0.001$; Fig. 1J) fat pads, across both diets ($p < 0.01$). HFD increased weight of the gonadal fat pads ($F = 16.44$, $p < 0.001$), although this effect reached statistical significance only in middle-aged animals ($p < 0.05$). HFD also increased weight of the RP fat pads ($F = 61.29$, $p < 0.0001$), across all age groups ($p < 0.05$). There were no significant interactions between age and diet on fat pad weights.

3.1.2. Body weight and adiposity: diet and testosterone treatment

We also examined the effects of CTL versus HFD on metabolic outcomes in middle-aged and aged rats that had received testosterone treatment throughout the experimental period (Fig. 1D–E). When examining plasma testosterone levels, we found that most animals that had not been treated with testosterone had levels below the detection threshold. Of those that did have detectable readings, young animals had an average of 0.256 ± 0.124 ng/mL; middle-aged had 0.298 ± 0.129 ng/mL, and aged animals had 0.166 ± 0.004 ng/mL. All testosterone-treated animals had detectable hormone levels, with middle-aged animals having an average of 0.382 ± 0.030 ng/mL and aged animals having 0.294 ± 0.024 ng/mL. To confirm efficacy of testosterone treatment, we compared weights of levator ani muscle, an established in vivo bioassay of androgens. Middle-aged rats on control diet treated with testosterone had significantly heavier levator ani than untreated middle-aged rats (0.920 ± 0.025 g vs. 0.630 ± 0.033 g, $p < 0.01$), a finding also observed in aged rats (0.916 ± 0.018 g vs. 0.730 ± 0.032 g, $p < 0.01$). Similar relationships were found for the androgen-responsive tissues prostate and seminal vesicles in both middle-aged and aged rats (data not shown). Thus, testosterone treatment increased plasma testosterone levels and weights of androgen-responsive tissues in both middle-aged and aged rats.

Although rats maintained on HFD showed higher mean body weights, there was no statistically significant main effect of diet on body weight across the 12 weeks in middle-aged animals (Fig. 1D) or in aged animals (Fig. 1E). However, there was a main effect of diet on the percent of weight gained across the treatment period ($F = 37.5$, $p < 0.0001$; Fig. 1F), and this effect was significant in both middle-aged ($p = 0.002$) and aged animals ($p < 0.0001$). Similarly, we found a significant main effect of diet on final body weight ($F = 14.38$, $p < 0.001$; Fig. 1F), which was significant only in aged animals ($p = 0.011$). In addition, there was a main effect of age on final body weight ($F = 7.98$, $p = 0.009$; Fig. 1G), which failed to reach statistical significance when examined across diets.

Levels of food intake showed significant main effects of both age ($F = 4.77$, $p = 0.038$; Fig. 1H) and diet ($F = 10.1$, $p = 0.004$). The main effect of age on food intake failed to reach statistical significance by post hoc test, and there were nonsignificant trends toward an effect of diet in middle-aged ($p = 0.058$) and aged animals ($p = 0.072$).

For measures of adiposity, we found significant main effects of diet on weights of both the gonadal ($F = 14.31$, $p < 0.001$; Fig. 1I) and RP ($F = 137.4$, $p < 0.001$; Fig. 1J) fat pads, with post hoc tests revealing that HFD significantly increased fat pad weights across both ages ($p < 0.05$). In addition, there was a significant effect of age on RP fat pad weight ($F = 8.37$, $p = 0.007$) with aged animals on HFD having greater fat pad weight than middle-aged animals ($p = 0.013$).

3.2. Glucose homeostasis

3.2.1. Glucose homeostasis: diet and aging

One of the major systems disrupted by DIO is glucose homeostasis. To examine the effects of diet and aging on this system, we performed glucose tolerance tests. We found a significant main effect of diet on glucose clearance in young animals ($F = 9.67$, $p = 0.008$; Fig. 2A) such that HFD-fed rats had higher glucose readings, although this effect reached statistical significance only at 15 minutes ($p < 0.001$) and 30 minutes ($p < 0.001$) after glucose bolus. Diet did not significantly affect rates of glucose clearance in middle-aged (Fig. 2B) and aged (Fig. 2C) animals. There were significant effects of both diet ($F = 8.27$, $p = 0.006$) and age ($F = 4.02$, $p = 0.026$) on GTT as measured by AUC (Fig. 2F), although both of these effects failed to reach statistical significance when examined across ages and diets, respectively. Finally, we assessed the extent to which animals were able to restore glucose homeostasis by calculating percent return to baseline glucose value (Fig. 2G). We found a significant main effect of age ($F = 7.09$, $p = 0.002$), but not of diet, on GTT return to the baseline. Between-group comparisons showed that aged animals on HFD were significantly impaired at returning to the baseline glucose levels compared with young animals on HFD ($p = 0.011$).

3.2.2. Glucose homeostasis: diet and testosterone treatment

In testosterone-treated animals, we found a significant main effect of diet on GTT in middle-aged animals ($F = 7.97$, $p = 0.014$; Fig. 2D) in which glucose levels were higher in HFD-fed animals at 30 minutes ($p = 0.025$). Diet did not significantly increase glucose levels in aged animals at any time point ($F = 0.48$, $p = 0.50$; Fig. 2E). GTT AUC did not differ by age, although there was a trend toward an increase in HFD groups that failed to reach statistical significance ($F = 3.54$, $p = 0.07$; Fig. 2F). Finally, neither age nor diet had a
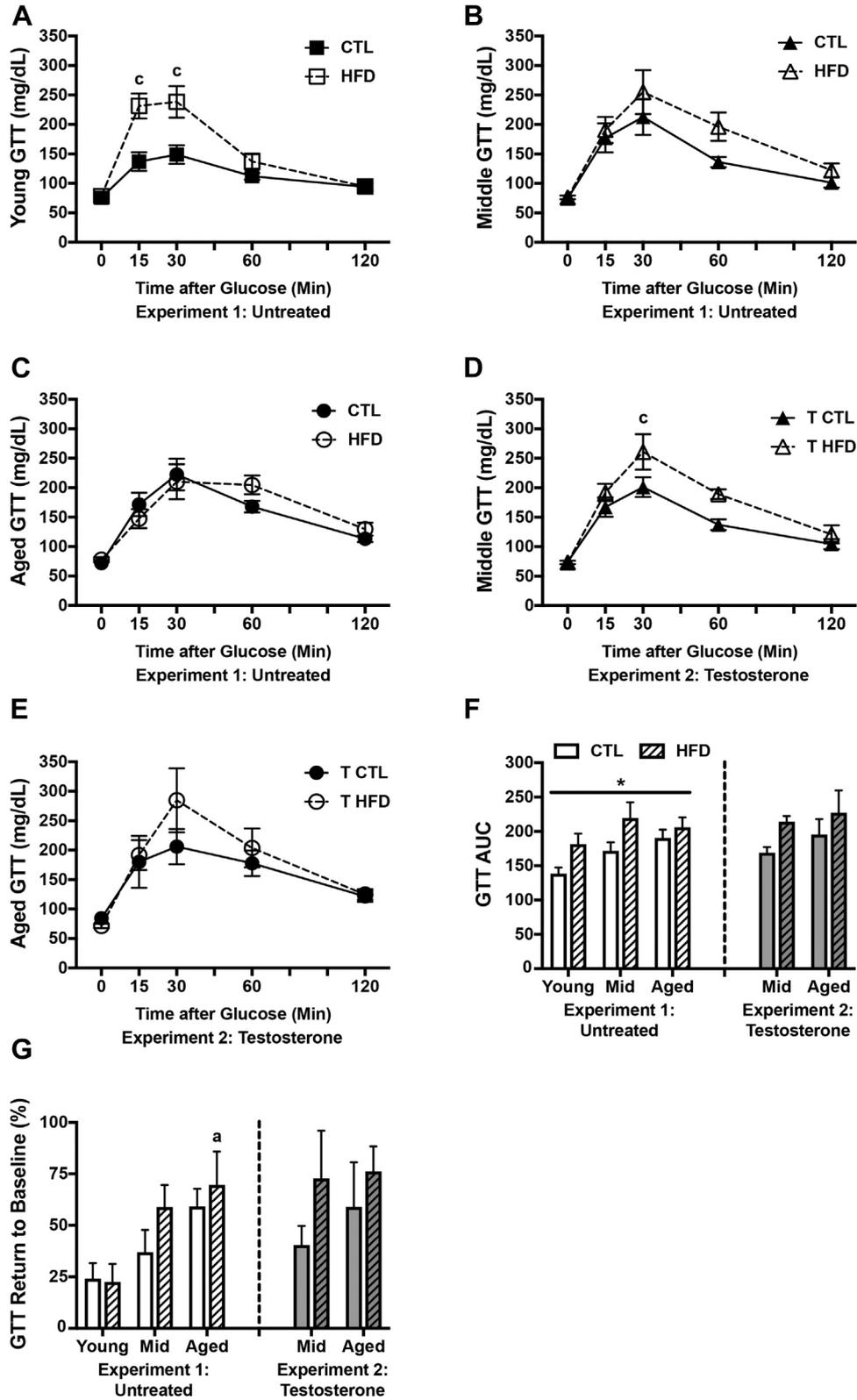
Fig. 2. Glucose homeostasis as assessed by glucose tolerance testing in young, middle-aged, and aged brown Norway rats on control or high-fat diet. (A-C) Blood glucose levels measured in mg/dL at baseline (0 minutes) and 15, 30, 60, and 120 minutes after administration of a glucose bolus in (A) young, (B) middle-aged, and (C) aged rats on control or high-fat diet. (D-E) Glucose levels in testosterone-treated (D) middle-aged and (E) aged rats fed control or high-fat diet and administered a glucose bolus. (F) Area under the curve (AUC) for the glucose tolerance test. (G) The extent to which animals returned to their baseline glucose levels after 120 minutes calculated as a percent change from their baseline level. For figures A–E, young animals are shown as squares, middle-aged animals as triangles, and aged animals as circles; control diet are open symbols, high-fat diet are closed symbols. For figures F–G, animals not treated with testosterone are shown in white (experiment 1), and animals treated with testosterone in gray (experiment 2); control diets are solid white or gray bars, high-fat diets are striped bars. (a) $p < 0.05$ relative to young rats in same diet condition. (c) $p < 0.05$ relative to control diet-fed rats of the same age. * $p < 0.05$ for main effect of age that does not reach statistical significance in post hoc tests.

significant effect on the extent to which glucose returned to baseline levels (Fig. 2G).

3.3. Peripheral effects of HFD

3.3.1. Peripheral effects of HFD: diet and aging

In addition to alterations in body weight and glucose homeostasis, HFD has numerous other effects on a variety of peripheral organs and systems. One change is increased plasma levels of the hormone leptin, which is released by adipose tissue. We observed a significant interaction between age and diet on plasma leptin levels ($F = 3.87$, $p = 0.029$; Fig. 3A) such that HFD was associated with increased leptin in middle-aged and aged rats ($p < 0.001$), but not in young rats. In addition, there was a main effect of age ($F = 31.6$, $p < 0.0001$) in which middle-aged animals on HFD and aged animals on either diet exhibited higher leptin than young adult rats ($p < 0.05$). There was a further significant increase in leptin levels in aged as compared to middle-aged animals ($p < 0.05$).

The liver is also vulnerable to the effects of HFD. We examined the gene expression of CD68 as a measure of hepatic inflammation, and of SREBP-1 and stearoyl-CoA desaturase, as measures of liver fatty acid metabolism and lipogenesis. We found a significant effect of age on CD68 mRNA levels ($F = 9.26$, $p = 0.0005$; Fig. 3B) in which aged animals had higher levels than young animals across both diets ($p < 0.05$). There was no significant effect of diet nor was there an interaction between age and diet on CD68 levels. Levels of the transcription factor SREBP-1 were significantly affected by age

($F = 5.10$, $p = 0.010$; Fig. 3C) with HFD-fed middle-aged and aged animals having decreased expression relative to young animals. In addition, there was a nonsignificant trend toward an effect of diet on SREBP-1 ($F = 3.99$, $p = 0.053$), but no significant interaction between age and diet. Finally, we found a significant effect of diet on mRNA levels of SCD1 ($F = 23.54$, $p < 0.0001$; Fig. 3D), which was significant only in middle-aged animals ($p < 0.0001$). Although there was a trend toward an interaction between age and diet on SCD1 levels, this failed to reach statistical significance ($F = 2.80$, $p = 0.073$), and there was no significant effect of age.

3.3.2. Peripheral effects of HFD: diet and testosterone treatment

Examination of leptin levels in testosterone-treated animals showed a significant interaction between age and diet ($F = 5.01$, $p = 0.03$; Fig. 3A). Post hoc tests revealed that the effect of age was only significant in HFD-fed rats ($p = 0.002$), whereas diet increased leptin levels at both ages ($p < 0.01$).

Assessment of gene expression in the liver revealed a nonsignificant trend toward increased CD68 levels with age ($F = 3.38$, $p = 0.077$; Fig. 3B) and no significant main effect of diet or interaction between diet and age. However, there were significant effects of diet on levels of both SREBP-1 ($F = 5.87$, $p = 0.02$; Fig. 3C) and SCD1 ($F = 15.57$, $p = 0.0005$; Fig. 3D). Between-group comparisons showed that for SREBP-1, HFD increased expression significantly in middle-aged animals only ($p = 0.02$), and for SCD1, HFD was associated with significantly decreased expression in both middle-aged and aged animals ($p < 0.05$).
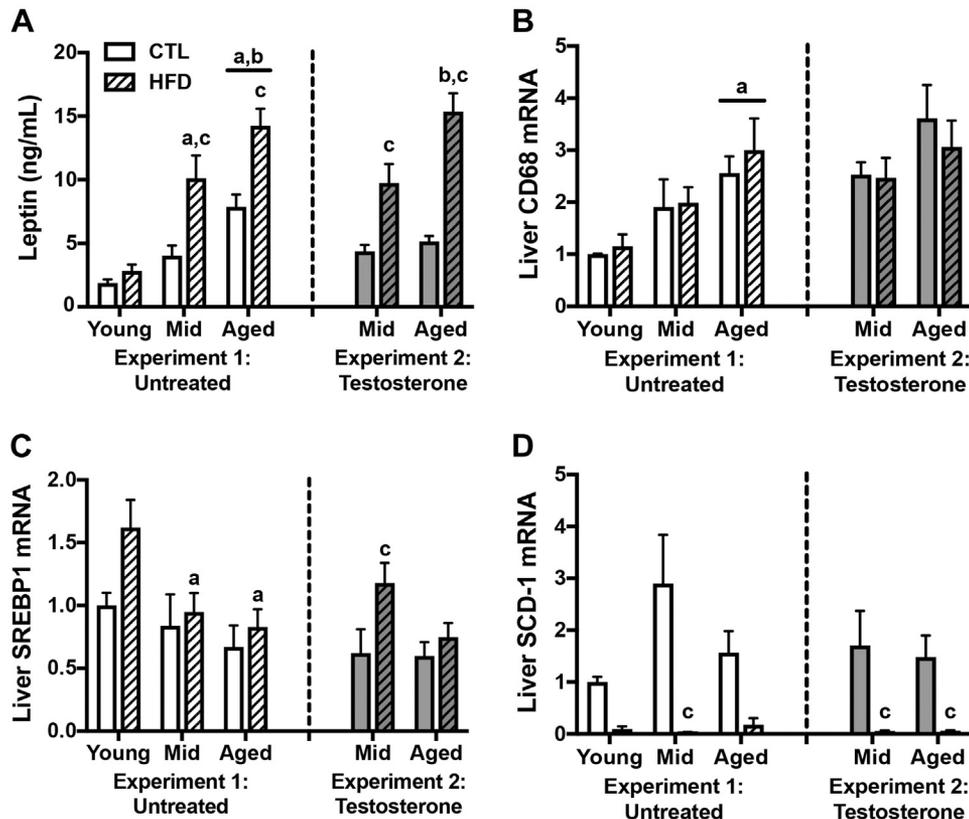

Fig. 3. Peripheral effects of control or high-fat diet in young, middle-aged, and aged brown Norway rats. (A) Plasma leptin levels measured in ng/mL in young, middle-aged, and aged rats on control or high-fat diet, at the end of the experimental period. Relative mRNA expression in the liver of (B) CD68, a macrophage marker; (C) SREBP1, a transcription factor regulating lipogenesis and glycolysis; and (D) SCD-1, a fatty acid metabolism enzyme, as determined by qPCR. Data show fold differences relative to the young rats on control diet. Animals not treated with testosterone are shown in white (experiment 1), and animals treated with testosterone in gray (experiment 2); control diets are solid white or gray bars, high-fat diets are striped bars. (a) $p < 0.05$ relative to young rats in same diet condition. (b) $p < 0.05$ relative to middle-aged animal in same diet condition. (c) $p < 0.05$ relative to control diet-fed rats of the same age.

3.4. Gliosis

3.4.1. Gliosis: diet and aging

Both HFD and advanced age increase activation states of microglia and astrocytes, the two neural cell types most responsible for regulating neuroinflammation. We examined the independent and interactive effects of diet and age on glial number and activation state. Microglial reactivity was assessed by morphology. Fig. 4A shows a resting microglial cell, characterized by numerous thin, branching processes (type 1). Reactive cells typically have enlarged rod-shaped cell bodies with fewer, thicker processes (type 2; Fig. 4B), or are ameboid (type 3; Fig. 4C). We first looked at microglial density in hippocampus and observed a significant main effect of age ($F = 22.95$, $p < 0.001$; Fig. 4D) in which middle-aged and aged animals across both diets had greater numbers of cells than young animals ($p < 0.01$). There was no significant main effect of diet on the density of microglia. We then examined the proportion of microglia with a reactive phenotype and found a significant interaction between age and diet ($F = 4.25$, $p = 0.021$; Fig. 4E) such that HFD was associated with increased microglial reactivity in young ($p < 0.001$) and middle-aged animals ($p < 0.05$), but not aged animals. In addition, there was a significant main effect of age ($F = 32.35$, $p < 0.001$) with middle-aged and aged animals having higher proportions of reactive microglia than young animals across both diets ($p < 0.01$).

We next performed parallel density and reactivity analyses with astrocytes. As shown in Fig. 5, nonreactive astrocytes have normally sized somas with several long, thin branches (Fig. 5A), whereas reactive astrocytes have enlarged somas and projections (Fig. 5B). We found no significant effect of either diet or age on astrocyte density (Fig. 5C). However, there were significant main effects of both age ($F = 7.41$, $p < 0.002$) and diet ($F = 9.28$, $p < 0.004$) on astrocyte reactivity (Fig. 5D). Between-group analyses revealed that aged animals had a higher percentage of reactive astrocytes than young animals ($p = 0.012$), but the effect of diet did not reach statistical significance when separated across ages.

3.4.2. Gliosis: diet and testosterone treatment

Next, we examined the relationship between aging and diet on inflammation outcomes in middle-aged and aged animals that received testosterone supplementation. We found no significant main effects or interactions on measures of microglial density (Fig. 4D) or the proportion of reactive microglia (Fig. 4E). Similarly, there were no significant effects of diet or age on astrocyte density (Fig. 5C). We found a significant main effect of age on the proportion of reactive astrocytes ($F = 7.24$, $p = 0.012$; Fig. 5D); however, this effect was not significant when examined separately across diets.

3.5. Inflammatory gene expression

3.5.1. Inflammatory gene expression: diet and aging

Another aspect of neuroinflammation that can be affected by HFD and aging is gene expression of proinflammatory and glial factors. We used quantitative PCR to examine gene expression of several such genes in both the hippocampus and hypothalamus. We found significant main effects of age on hippocampal gene expression of IL-1β ($F = 5.41$, $p = 0.002$; Fig. 6A), CD68 ($F = 25.85$, $p < 0.001$; Fig. 6B), and GFAP ($F = 8.19$, $p = 0.001$; Fig. 6C). Between-group analyses revealed that (1) on CTL diet, aged animals had greater levels of IL-1β than young animals ($p = 0.029$); (2) aged animals had higher expression of CD68 than both young and middle-aged animals on CTL diet and had higher levels than young animals on HFD ($p < 0.001$), and (3) aged animals had higher levels of GFAP than young animals on either diet ($p < 0.05$). Neither diet nor the interaction between age and diet significantly affected expression levels of these genes in the hippocampus.

Hypothalamic gene expression of IL-1β was not significantly affected by either age or diet (Fig. 6D). There was a significant interaction between age and diet on levels of CD68 ($F = 3.68$, $p = 0.034$; Fig. 6E) and a trend toward an interaction effect on GFAP levels ($F = 2.98$, $p = 0.062$; Fig. 6F), with diet increasing gene expression only in aged animals ($p < 0.01$). In addition, there was a
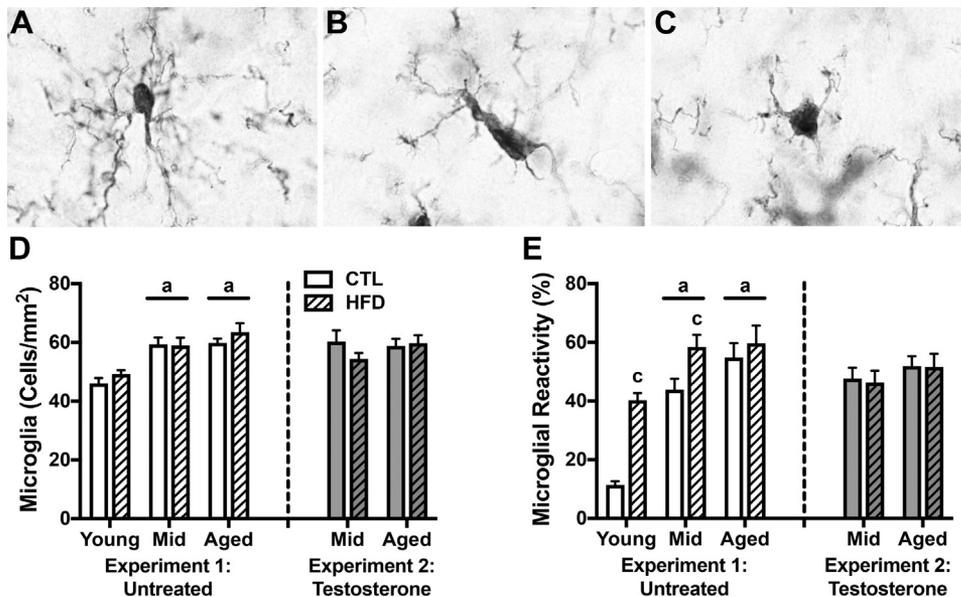

Fig. 4. Microglia number and morphological status as assessed by IBA-1 immunohistochemistry in young, middle-aged, and aged brown Norway rats across dietary and testosterone treatments. (A-C) Representative images of microglial morphology. Scale bar = 10 μm. (A) A type 1 or resting cell characterized by a small cell body with numerous, ramified projections. Reactive cells are either (B) type 2 cells with rod-shaped cell bodies and fewer, thicker processes, or (C) ameboid cells with no branches or filopodia. (D) Densities of IBA-1 immunoreactive microglia in young, middle-aged, and aged rats on control or high-fat diet were quantified in the hippocampus. (E) Percentages of reactive microglia (types 2 and 3) were quantified in the hippocampus. Animals not treated with testosterone are shown in white (experiment 1), and animals treated with testosterone in gray (experiment 2); control diets are solid white or gray bars, high-fat diets are striped bars. (a) $p < 0.05$ relative to young rats in same diet condition. (c) $p < 0.05$ relative to control diet-fed rats of the same age.
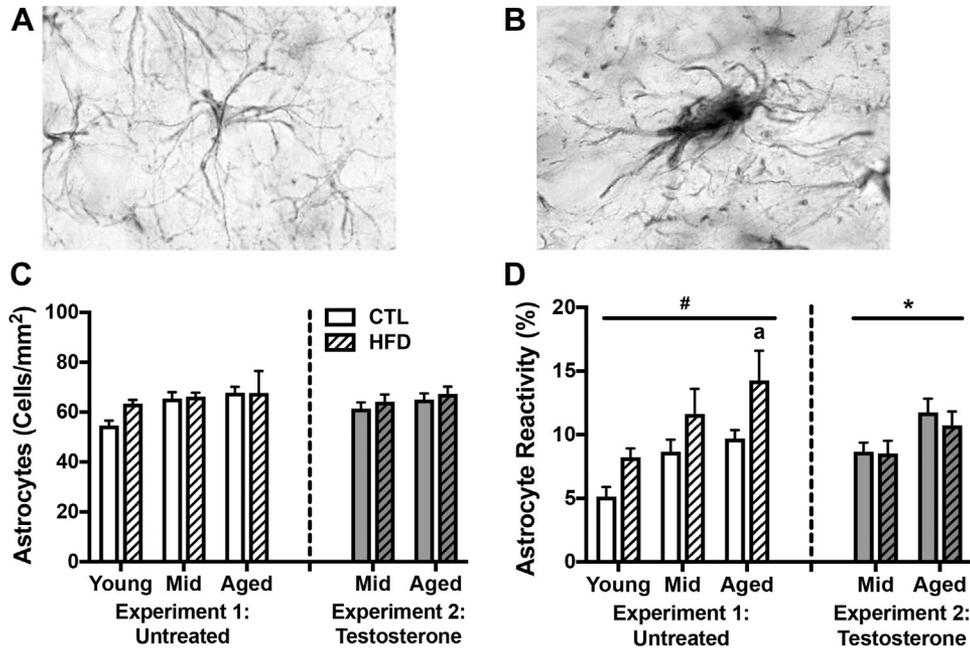
Fig. 5. Astrocyte number and morphological status as assessed by GFAP immunohistochemistry in young, middle-aged, and aged brown Norway rats across diet and testosterone treatments. (A-B) Representative images of astrocyte morphology. Scale bar = 10 μm. (A) Nonreactive astrocytes have normally sized cell bodies and thin projections. (B) Reactive astrocytes have enlarged somas and thicker processes. (C) Densities of GFAP-immunoreactive astrocytes and (D) percentages of reactive astrocytes were quantified in hippocampus of young, middle-aged, and aged rats on control or high-fat diet and untreated or treated with testosterone. Animals not treated with testosterone are shown in white (experiment 1) and animals treated with testosterone in gray (experiment 2); control diets are solid white or gray bars, high-fat diets are striped bars. (a) $p < 0.05$ relative to young rats in same diet condition. * $p < 0.05$ for main effect of age that does not reach statistical significance in post hoc tests. # $p < 0.05$ for main effect of diet that does not reach statistical significance in post hoc tests.
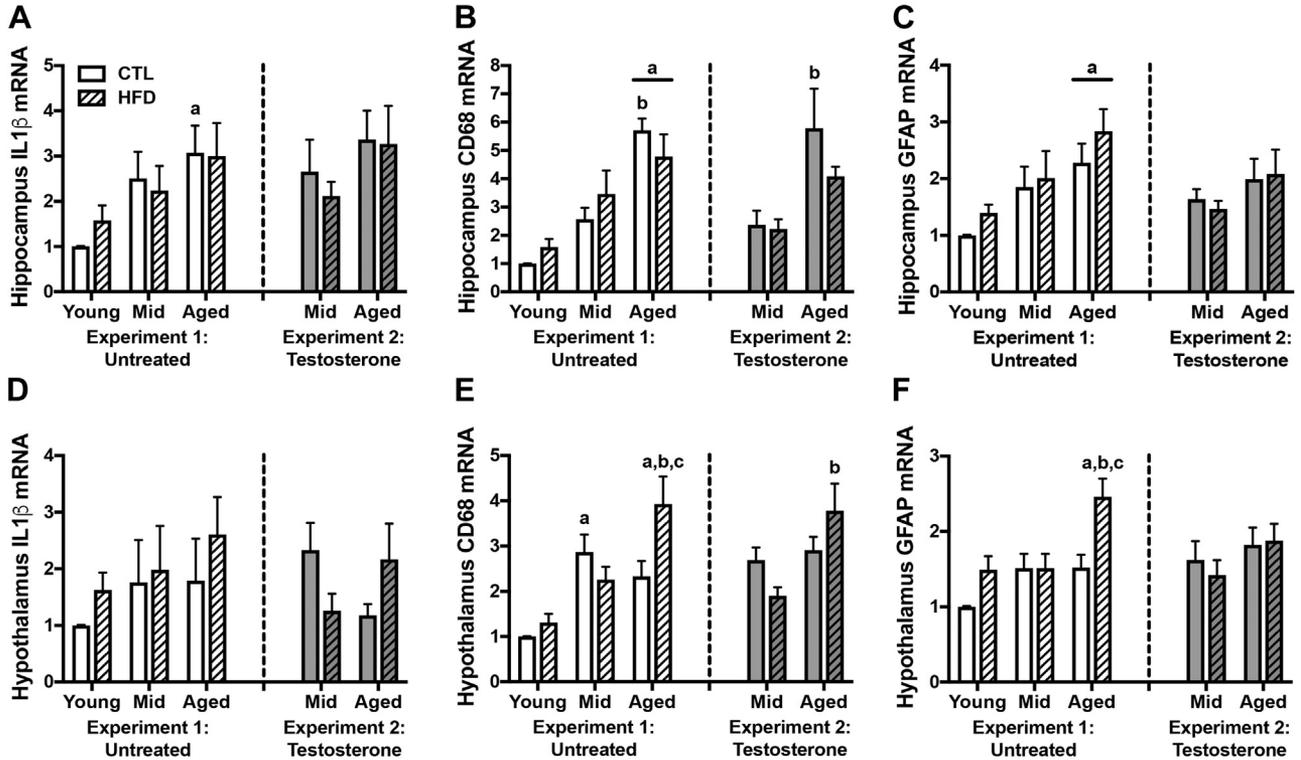

Fig. 6. Inflammatory gene expression in young, middle-aged, and aged brown Norway rats across diet and testosterone treatments. (A-C) Relative mRNA levels in the hippocampus were determined by qPCR for (A) IL1β, a proinflammatory cytokine; (B) CD68, a microglia/macrophage marker; and (C) GFAP, an astrocyte marker. (D-F) Relative mRNA levels for (D) IL1β; (E) CD68; and (F) GFAP were also determined in the hypothalamus. Data show fold differences relative to the young rats on control diet. Animals not treated with testosterone are shown in white (experiment 1) and animals treated with testosterone in gray (experiment 2); control diets are solid white or gray bars, high-fat diets are striped bars. (a) $p < 0.05$ relative to young rats in same diet condition. (b) $p < 0.05$ relative to middle-aged animal in same diet condition. (c) $p < 0.05$ relative to control diet-fed rats of the same age.

significant main effect of age on levels of CD68 ($F = 12.35$, $p < 0.001$) and GFAP ($F = 7.69$, $p = 0.002$). Post hoc tests revealed that on CTL diet, middle-aged animals had higher levels of CD68 than young animals ($p < 0.01$), and on HFD, aged animals had higher levels of CD68 and GFAP than both young and middle-aged animals ($p < 0.05$).

3.5.2. Inflammatory gene expression: diet and testosterone treatment

Among testosterone-treated rats, we found no significant effects of age, diet, or their interaction on expression of IL-1β (Fig. 6A) and GFAP (Fig. 6C) in the hippocampus. However, there was an effect of age on levels of hippocampal CD68 ($F = 13.21$, $p = 0.001$; Fig. 6B), with post hoc tests showing that aged animals on CTL diet had higher CD68 than matched middle-aged animals ($p = 0.006$). Hypothalamic gene expression showed a significant interaction between age and diet on levels of IL-1β ($F = 6.29$, $p = 0.018$; Fig. 6D), although this interaction failed to achieve statistical significance in post hoc analysis. Likewise, we found a significant interaction between age and diet on CD68 expression in the hypothalamus ($F = 5.55$, $p = 0.026$; Fig. 6E), in which aged animals had higher levels than middle-aged animals, but only on HFD ($p = 0.002$). Finally, levels of hypothalamic GFAP did not differ by age, diet, or their interaction (Fig. 6F).

3.6. Behavioral and neurogenesis outcomes

3.6.1. Behavioral and neurogenesis outcomes: diet and aging

To determine whether age and diet modulate cognitive performance, we examined behavior on the Barnes maze, a test of spatial reference learning and memory. We first assessed learning behavior by examining the latency to reach the escape box on each trial during training. Fig. 7A–C show the latency averaged across the 3 trials per day. For all age and diet groups, the animals showed significant learning, as indicated by shorter latencies to reach the escape box on days 2–4, than on day 1 of training ($p < 0.05$). However, rates of learning differed across groups. There was a significant main effect of age across CTL diet animals ($F = 7.02$, $p = 0.004$), with aged animals being slower at locating the escape platform than both young ($p = 0.005$) and middle-aged animals ($p = 0.045$). However, this effect is likely due to slower locomotion in aged animals, rather than to a learning disability, as all animals were able to locate the escape box within 30 seconds by the last training day. There were no significant effects of diet in any age group, suggesting that diet did not impair ability to learn the location of the escape box.

When examining performance on the probe trial, we found a significant main effect of age ($F = 6.79$, $p = 0.003$; Fig. 7F) such that on HFD, young animals approached the correct hole more frequently than either middle-aged ($p = 0.002$) or aged animals ($p = 0.021$). Likewise, when looking at the percent of correct hole approaches (Fig. 7G), we found a trend toward a main effect of age with younger age exhibiting more correct hole approaches ($F = 2.54$, $p = 0.089$), although this failed to reach statistical significance. Neither diet nor the interaction between age and diet had significant effects on the number or percent correct hole approaches. There was a significant main effect of diet on errors, where rats fed HFD approached more incorrect holes ($F = 4.42$, $p = 0.041$; Fig. 7H), although this was not statistically significant when examined separately across the ages. Finally, we found a significant main effect of diet on path length, with greater distance traveled in HFD-fed rats ($F = 4.54$, $p = 0.039$; Fig. 7I), that did not reach statistical significance when examined across the age groups. However, our data show a greater percent increase in path length associated with HFD in middle-aged (~132%) rats than in young (~70%) and aged
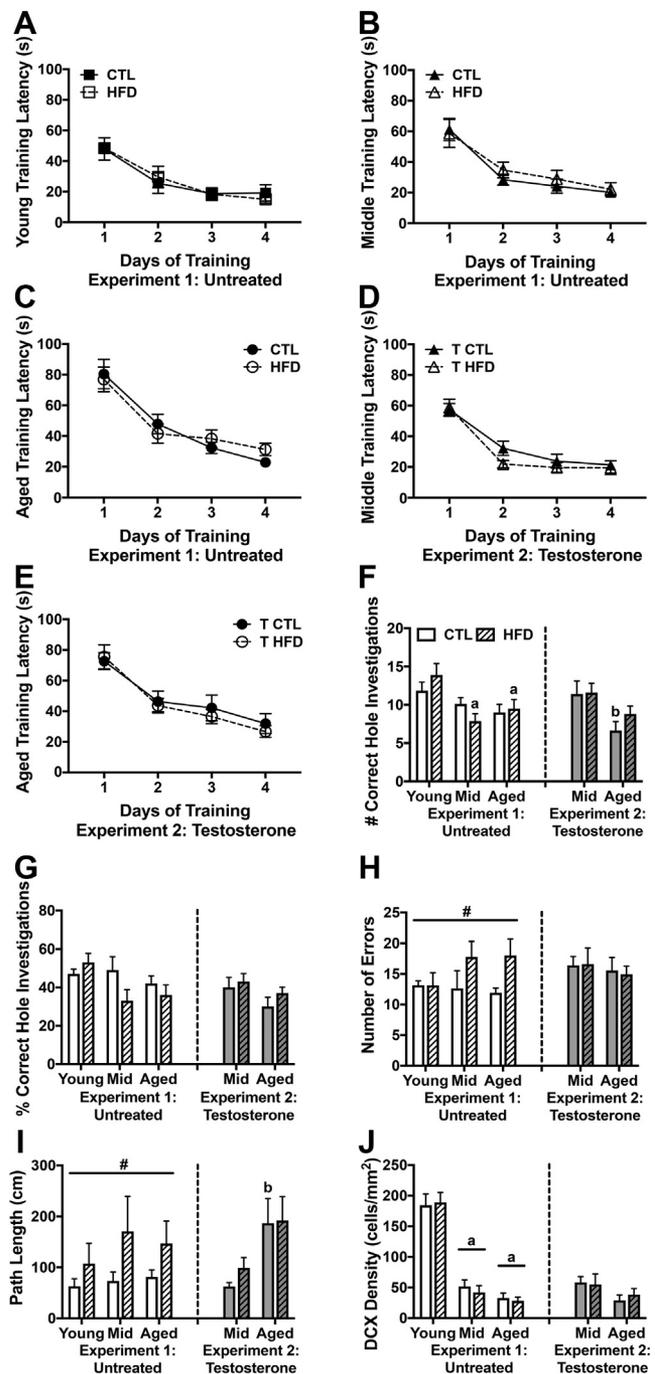

Fig. 7. Effects of diet and testosterone on behavioral performance in the Barnes maze and on neurogenesis in young, middle-aged, and aged brown Norway rats. (A-C) Average latency to locate the escape box over the course of 4 days of training in (A) young, (B) middle-aged, and (C) aged male brown Norway rats maintained on control and high-fat diet. (D-E) Average latency to locate the escape box in testosterone-treated (D) middle-aged and (E) aged rats fed control or high-fat diet. (F) The number and (G) percentage of correct hole visits during the Barnes maze probe trial, 48 hours after the last training trial. (H) The number of errors made during the probe trial and (I) the distance traveled to locate the escape box for the first time during the probe trial. (J) Neurogenesis as measured by the density of DCX-expressing cells in the dentate gyrus. Animals not treated with testosterone are shown in white (experiment 1) and animals treated with testosterone in gray (experiment 2); control diets are solid white or gray bars, high-fat diets are striped bars. (a) $p < 0.05$ relative to young rats in same diet condition. (b) $p < 0.05$ relative to middle-aged animal in same diet condition. # $p < 0.05$ for main effect of diet that does not reach statistical significance in post hoc tests.

(∼80%) rats, suggesting that middle-aged animals were most susceptible to the effects of HFD. Neither age nor the interaction between age and diet had significant effects on errors or path length.

To examine neurogenesis, we analyzed the density of DCX-expressing cells in the dentate gyrus. As shown in Fig. 7J, there was a main effect of age ($F = 98.65$, $p < 0.0001$) in which young animals had a higher density of DCX-expressing cells than middle-aged and aged animals across both diets ($p < 0.0001$). There was no significant effect of diet nor of an interaction between diet and age on the density of DCX expression.

3.6.2. Behavioral and neurogenesis outcomes: diet and testosterone treatment

Behavior in the Barnes maze was also assessed in testosterone-treated animals. Both middle-aged (Fig. 7D) and aged (Fig. 7E) rats located the escape box more quickly on day 2 and all subsequent days of training than on day one ($p < 0.05$). There was a main effect of age for both CTL diet ($F = 5.61$, $p = 0.029$) and HFD animals ($F = 14.6$, $p = 0.001$) with aged animals taking longer to find the escape box. There was no significant effect of diet nor was there an interaction between age and diet on training latency.

Examination of probe trial performance yielded results similar to those observed in animals not treated with testosterone. That is, we found a significant main effect of age ($F = 8.68$, $p = 0.005$; Fig. 7F) with aged animals on CTL diet having less correct hole approaches than middle-aged animals ($p = 0.026$). Furthermore, there again was a trend toward reduced correct hole investigations with age ($F = 3.34$, $p = 0.076$; Fig. 7G). However, neither diet nor the interaction between age and diet had significant effects on the number or percent of correct hole investigations. There were no significant main or interactive effects of aging and diet on the number of probe trial errors (Fig. 7H). We found a significant main effect of age on path length ($F = 9.14$, $p = 0.005$; Fig. 7I) such that in CTL diet animals, aged rats traveled further to locate the correct hole than did middle-aged animals ($p = 0.04$). There was no main effect of diet nor was there an interaction between age and diet on path length.

When examining DCX-labeled cell density, we found a nonsignificant trend toward an effect of age ($F = 3.73$, $p = 0.064$; Fig. 7J), but neither diet nor the interaction between diet and age had a significant effect on DCX density.

3.7. Correlations

We examined correlations between various metabolic and inflammatory markers and performance in the Barnes maze, specifically in animals from experiment 1, results of which are shown in Table 2. As expected, metabolic outcome measures of adiposity, leptin, and GTT AUC are highly correlated with each other and with

food intake. The percent change in body weight correlates with food intake but not with any other metabolic outcome measure or with any glial or behavioral outcome. There is also high correlation between the glial measures of microglia and astrocyte activation and the behavioral outcome measures of correct hole entries, errors, and path length.

Metabolic measures are highly correlated with both measures of glial activation but show nonsignificant correlations with behavioral outcomes, except for a trend toward significance in adiposity and leptin on correct hole entries. However, microglial activation is significantly correlated with both correct hole entries and path length, highlighting the role of microglia in this context. Thus, metabolic outcomes were correlated with glial activation, which was then correlated with behavioral outcomes.

3.8. Effects of testosterone treatment

The main goals of this study were to examine (1) interactions between age and diet and (2) the potential of testosterone to modulate diet outcomes during aging. Although not a planned goal of the study, the data set does provide an opportunity to assess the effects of testosterone treatment in middle-aged and aged rats across diets. Because different sets of animals were used in experiments 1 and 2, we were unable to directly compare by statistical analyses the effects of testosterone treatment. Nonetheless, some descriptive examination of the data is possible.

We observed only a few positive effects of testosterone treatment upon comparison of experiment 1 middle-aged and aged rats without testosterone treatment versus age- and diet-matched rats with testosterone treatment. Specifically, testosterone was associated with a ∼20% reduction in gonadal fat mass (Fig. 1I) and a ∼50% reduction in levels of plasma leptin (Fig. 3A) in aged rats. Testosterone had negligible effects in CTL-fed middle-aged animals, with only an ∼8% decrease in gonadal fat mass and no appreciable change in leptin levels, suggesting that any testosterone protection against age-related metabolic impairments may be limited to advanced ages.

Testosterone treatment did not alter inflammatory outcomes in CTL-fed rats, but appears to have yielded subtle reductions in gliosis. In untreated middle-aged animals, HFD was associated with ∼25% increases in both microglial (Fig. 4E) and astroglial (Fig. 5D) activated morphology. In testosterone-treated middle-aged animals, HFD was not associated with increases in microglial and astroglial reactivity, suggesting that testosterone treatment diminished the effect of HFD in middle-aged animals. Similar effects of were observed in aged animals.

In the Barnes maze, testosterone treatment in CTL-fed animals had opposite effects at middle age and old age. That is, during the probe trial, testosterone was associated with a ∼17% decrease in

Table 2
Correlations among metabolic, inflammatory, and behavioral outcomes

Measure	kCal	Body weight (% change)	Adiposity (RP fat)	Leptin	GTT AUC	Microglia activation	Astrocyte activation	Correct hole entries	Errors	Path length
kCal	——									
Body weight (% change)	0.35*	——								
Adiposity (RP fat)	0.72****	0.11	——							
Leptin	0.77****	0.07	0.91****	——						
GTT AUC	0.17	−0.01	0.40**	0.37*	——					
Microglia activation	0.49***	−0.08	0.63****	0.59****	0.48***	——				
Astrocyte activation	0.49***	0.03	0.53****	0.39**	0.12	0.39**	——			
Correct entries	−0.13	0.23	−0.28^T	−0.28^T	−0.24	−0.38**	−0.21	——		
Errors	0.11	0.18	0.21	0.22	0.17	0.23	0.14	−0.45**	——	
Path length	0.10	0.09	0.09	0.17	0.21	0.33*	0.14	−0.28^T	0.66****	——

Data are presented as Spearman r values. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$; $^T p < 0.07$.
Key: AUC, area under the curve; GTT, glucose tolerance test; RP, retroperitoneal.

path length in middle-aged animals, but a ~56% increase in aged animals (Fig. 7I), suggesting that it negatively affected cognitive performance in 26-month-old rats. When examining HFD-associated increases in path length in middle-aged animals, we found a ~130% increase in untreated rats, which was reduced to a ~60% increase in testosterone-treated rats, suggesting that testosterone treatment is associated with modest protection from the effects of HFD on behavioral performance at middle age.

4. Discussion

A primary goal of this study was to determine whether HFD differentially affected various metabolic, inflammatory, and cognitive outcomes across advancing age. We found that, regardless of diet, aging had the strongest adverse effects on a number of measures, corroborating the idea that aging is the central driving force for negative health consequences and disease. For example, aging was associated with increased body weight, adiposity, and leptin levels, indicating a general shift toward worse metabolic outcomes with age. Consistent with our findings, previous studies in rodent models also showed increased adiposity with age (Larkin et al., 2001; Wolden-Hanson et al., 1999) as well as greater weight gain in response to HFD in older animals (Erdos et al., 2011). Moreover, we found that both middle-aged and aged animals had increased microglial reactivity, and aged animals showed higher expression of several proinflammatory factors. These findings of increased inflammation with age are well documented in humans (Freund et al., 2010) and rodent models (Sierra et al., 2007; Wu et al., 2007).

In addition to aging, we found generally modest effects of HFD on metabolic, inflammatory, and behavioral changes. As expected, HFD was associated with a number of adverse metabolic results, including increases in body weight, adiposity, and leptin levels as well as reduced glucose clearance in young animals. Moreover, HFD increased microgliosis without significantly affecting astrocyte reactivity or cytokine expression. However, because animals were not perfused, it is possible that cytokine expression is affected by blood and leukocyte infiltration, although the changes associated with this would be expected to be rather small (York et al., 2012). Although some prior studies have shown HFD-associated increases in both glial reactivity and cytokine levels (Pistell et al., 2010), others show changes in only a subset of neuroinflammatory markers (Setti et al., 2015) or only in select brain regions (Guillemot-Legris et al., 2016). We also found that overall HFD was associated with worsened spatial memory performance, which is consistent with other reports (Molteni et al., 2002). Thus, age and diet were both independently associated with a range of adverse metabolic, inflammatory, and behavioral changes.

It is important to note that we observed only moderate increases in body weight in response to HFD. Comparison of mean final body weights showed that HFD was associated with ~12% increase in body weight in young animals, and a ~7% increase in middle-aged and aged animals, over the three-month period. When evaluated as percent change in individual animals over the course of the study, HFD in middle-aged and aged animals yielded net increases in body weight of ~7 and ~12%, respectively. For comparison, for an average height man with normal BMI, an increase of 9% body weight would correspond to an elevation in BMI of ~2 units. Thus, the relatively modest effects of HFD may reflect the mild increases in body weight and adiposity yielded by this HFD. By this perspective, the HFD-induced effects may reflect long-term exposure to an obesogenic diet rather than obesity. It is of interest that recent observations show that short-term HFD exposure is also associated with deleterious neural effects that are exacerbated in aged animals (Spencer et al., 2017). Perhaps unexpectedly, we observed that increasing age exerted a much stronger effect on

body weight than HFD. In rats on control diet, mean body weight progressively increased from young adulthood to middle age and old age, such that there was an overall ~33% weight increase with aging. In line with the age-related increase in body weight, aging was also associated with greater food consumption, with aged rats consuming a greater amount of kcal per day across both diets. The observed age-related increases in body weight and adiposity may reasonably have contributed to the effects of age on metabolic, inflammation, and neurogenesis measures.

In addition to their independent effects, age and diet interactively affected several outcomes. For example, we observed significant effects of aging on measures of ability to return to the baseline in GTT, astrocyte reactivity, expression of hypothalamic CD68 and GFAP, and the number of correct hole visits in the Barnes maze probe, specifically in HFD-fed animals. Similar findings of age and diet interactions were recently demonstrated in the context of short-term HFD exposure in which only aged animals on HFD showed cognitive impairments (Spencer et al., 2017). Conversely, it has also been shown that obesity impairs cognitive performance in mice exposed to HFD during adolescence, but not in adulthood (Boitard et al., 2012), suggesting that the brain may be especially vulnerable to effects of diet during both early and late life. Collectively, these findings support the "two-hit hypothesis" that has been proposed for a number of diseases (Hawkes et al., 2007; Zhu et al., 2004) and consequences of age-related inflammation (Franceschi et al., 2000). According to this position, aging may act as the first hit that increases vulnerability of several systems to the adverse effects associated with a second challenge, like HFD. Such interactive effects between various risk factors may have important implications for prevention and/or treatment of diseases.

Interestingly, we found that middle-aged animals were more vulnerable to some effects of HFD. More specifically, HFD induced relatively more robust changes in body weight, adiposity, leptin levels, liver SCD1 expression, and microglial activation in middle-aged animals than in both young and aged animals, which exhibited HFD-induced effects in some but not all of these measures. Moreover, although not statistically significant, the effects of HFD on cognitive performance in the Barnes maze appeared strongest in middle-aged animals. A number of studies in the human literature have demonstrated increased risk for dementia (Emmerzaal et al., 2015; Fitzpatrick et al., 2009) and accelerated brain aging (Ronan et al., 2016) in response to midlife obesity. Effects of obesity at midlife have thus far not been thoroughly investigated in experimental models. One study found that middle-aged rats showed memory impairments, loss of dendrites, and microglial activation in response to a diet high in cholesterol and saturated fatty acids (Granholm et al., 2008). However, young adult and aged animals were not included in this study to compare whether dietary effects were particularly harmful in middle age. Our study is unique in its focus on comparing effects of HFD across animals in young adulthood, middle age, and advanced age. Although we did not directly examine how HFD in middle-aged rats affected outcomes in advanced age, our data suggest that the pronounced effect of HFD at middle age may serve as a catalyst that pushes the aging trajectory to poorer outcomes later in life.

A second goal of this study was to evaluate the ability of testosterone treatment to attenuate consequences of HFD in middle-aged and aged rats. Overall, we found very limited effects of testosterone treatment on the range of metabolic, inflammatory, and neural outcomes assessed in this study. Comparisons across the two experiments showed that some consequences of HFD observed in the absence of testosterone (experiment 1) were less robust in the presence of testosterone (experiment 2). Specifically, in experiment 2, HFD was not associated with increase in microglial activation in middle-aged rats nor elevated astrocyte activation and

GFAP expression in aged animals. These observations are consistent with a prior report showing testosterone decreases age-related increases in GFAP expression (Day et al., 1998).

In terms of cognitive function, we found only nonsignificant trends of improved function with testosterone in middle-aged rats. However, testosterone treatment was associated with reduced spatial memory in aged rats maintained on control diet, suggesting an unfavorable effect of testosterone in these animals. Although some studies have shown improved cognition with testosterone treatment in men (Cherrier et al., 2005; Janowsky et al., 1994) and rodents (Frye and Seliga, 2001), many studies have failed to show an association (Emmelot-Vonk et al., 2008; Haren et al., 2005; Lu et al., 2006; Puts et al., 2010; Resnick et al., 2017) or have found deleterious effects of testosterone under conditions of oxidative stress (Cunningham et al., 2014). Moreover, although levels of neurogenesis in adult animals have been shown to be decreased with obesity (Lindqvist et al., 2006; Park et al., 2010) and increased with testosterone (Spritzer and Galea, 2007), we observed a significant reduction in DCX-expressing cells in middle-aged and aged animals that did not differ with diet or testosterone treatments. Within the limits of our assessments, our observations in male rats are consistent with much of the human literature in suggesting that testosterone does not have significant neural benefits during aging. Indeed, despite numerous potential advantages of testosterone therapy (Bassil et al., 2009), our overall findings align well with emerging evidence of limited positive outcomes of testosterone in aging men (Snyder et al., 2018).

Although the exact pathways linking obesity to cognitive decline and dementia are unknown, one hypothesis is that metabolic impairments drive neural impairment. It is well established that there are changes in metabolic factors in the AD brain (de la Monte and Wands, 2008). There is evidence that insulin deficiency and resistance in the brain are associated with cognitive impairment and AD (Craft, 2005), and reductions in insulin signaling are exacerbated as AD progresses (Rivera et al., 2005). Moreover, high plasma insulin can increase levels of Aβ42 in cerebrospinal fluid of healthy older adults (Watson et al., 2003), and there is evidence that improving insulin sensitivity in AD patients is associated with better cognitive outcomes (Reger et al., 2006; Watson et al., 2005). However, we found that neither impairments in GTT nor adiposity significantly correlated with behavioral performance, although there was a nonsignificant trend toward a correlation between leptin levels and the number of correct hole entries. Interestingly, a high-density of leptin receptors is found in the hippocampus and leptin resistance is tied to deficits in synaptic plasticity and hippocampal-dependent behaviors (Harvey et al., 2006; Irving and Harvey, 2014; Münzberg and Morrison, 2015). Interestingly, leptin receptors are found on astrocytes, and leptin can affect astrocyte morphology and function (García-Cáceres et al., 2011; Kim et al., 2014), suggesting that leptin may contribute to relationships between HFD and gliosis. Although our findings suggest that metabolic impairments largely did not predict behavioral outcomes, we did not specifically examine metabolic pathways in the brain and thus cannot rule out their potential effects.

Although there is a role for metabolic factors in regulation of cognitive function, there also is increasing evidence for a role of glial cells. For example, gliosis is hallmark of both aging and AD and has been proposed as a possible mechanism underlying cognitive impairment and AD pathogenesis (Blasko et al., 2004; Glass et al., 2010). In fact, several studies clearly point to a role for inflammation rather than metabolic changes in the effects of obesity on the brain. For instance, a prior study showed that 8 weeks of HFD resulted in cognitive deficits, decreased synaptic spines, and microglial activation, but no changes in insulin or glucose levels (Bocarsly et al., 2015). The finding that just 3 days of HFD exposure

leads to cognitive impairments in aged rats (Spencer et al., 2017) also suggests that these changes are likely independent of adiposity and metabolic outcomes. Inflammatory and glial changes, however, can be observed after just 1 day of HFD feeding and before metabolic changes occur (Thaler et al., 2012). In fact, inflammation may be driving the metabolic changes seen with HFD feeding and obesity (Maldonado-Ruiz et al., 2017), as central administration of proinflammatory cytokines has been shown to impair peripheral insulin signaling (Arruda et al., 2011), and exacerbate the effects of HFD feeding (Oh-I et al., 2010). Our findings of significant correlations between microglial reactivity and behavioral impairments support the role of gliosis as a central factor in mediating the effects of obesity on the brain. Interestingly, we found that astrocyte activation did not significantly correlate with behavioral outcomes nor were they significantly increased in response to HFD, which may suggest that microglia have a greater role in this relationship. This is supported by other studies showing a central role for microglia in obesity (De Luca et al., 2016; Tucsek et al., 2014), including the finding that HFD causes synaptic stripping by microglia (Hao et al., 2016). Taken together, these findings suggest that inflammation is an early consequence of HFD and may drive many of the adverse changes associated with obesity.

This study has limitations that should be addressed in future research. First, the design of this study limits our interpretations of testosterone actions. Experiments 1 and 2 were designed to assess the effects of aging and testosterone treatment during aging, respectively, on the neural and metabolic outcomes induced by HFD. The independent design of the two experiments precludes direct statistical comparison of groups between experiments 1 and 2. Thus, the data limit the ability to analyze how testosterone may have affected age-related changes in the absence of HFD beyond qualitative comparisons. Second, due to limited sensitivity of the testosterone assay, we were unable to determine whether age and/or HFD affected endogenous plasma testosterone levels, although we did confirm efficacy of testosterone treatment. The choice of brown Norway rat may also have impacted the magnitude of the observed HFD-induced effects. Although an excellent rodent model of male reproductive aging (Gruenewald et al., 2008; Wang et al., 1993, 2002), brown Norway rats are a relatively lean strain in comparison to Sprague-Dawley rats (Gordon et al., 2016) and show more modest weight gain in response to HFD than strains such as Sprague-Dawley or Wistar rats (Buettner et al., 2007). Thus, the interaction between HFD and aging may be more pronounced in other animal models. Finally, although groups in experiment 2 were controlled for surgeries, anesthesia is known to effect neural outcomes (Zhu et al., 2010) and may have affected findings differently across experiments 1 and 2.

Collectively, our results show that the effects of HFD vary across increasing ages, and that middle age may present a particularly vulnerable period. Importantly, the rate of obesity peaks at middle age, with ∼43% of U.S. adults between the ages of 40 and 59 years classified as obese (Hales et al., 2017). This represents a large population whose risk for a number of age-related diseases, including AD, could be mitigated by lifestyle changes. Midlife likely presents an especially important time point for protective interventions including testosterone treatment in men, as interventions at midlife are well positioned to alter the trajectory of age-related diseases. However, consistent with an increasing literature on effects of testosterone in aging men (Emmelot-Vonk et al., 2008; Snyder et al., 2018; Storer et al., 2017), our findings in aging rats do not indicate robust benefits of testosterone supplementation. Determining the underlying pathological processes that are driving adverse effects of obesity is critical. Findings in our study suggest an important role for gliosis. In fact, it may be the case that factors like obesity are especially harmful at middle age because of their role in driving

inflammatory processes, as it was recently shown that inflammation in middle age is associated with greater loss of brain volume later in life (Walker et al., 2017). Thus, it will be important to explore various risk factors that drive adverse aging trajectories in midlife, as well as the mechanisms underlying their effects, with the ultimate goal of identifying strategies and interventions to alter these trajectories.

Disclosure statement

The authors have no actual or potential conflicts of interest.

Acknowledgements

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